culosis, which makes it a useful probe for diagnostic and epidemiological purposes. The final composition of the PCR mix was the same as in the previous case. The PCR conditions were preheating at 94°C for 5 min, then 40 cycles at 94°C for 2 min, 68°C for 2 min, 72°C for 2 min, and then 72°C for 10 min.

Determination of sensitivity

To determine the sensitivity of *M. tuberculosis* detection, 10-fold serial dilutions (1 ng to 10 fg) of H37Rv DNA (kindly provided by Dr I Sugawara, Research Institute of Tuberculosis, Tokyo, Japan) were performed using T4-T5 primers. The final composition and PCR conditions were the same as for amplifying IS*6110*.

All amplification products were detected on 1.5% agarose gel in 1 × TAE buffer stained with ethidium bromide and visualised by ultraviolet transillumination.

Control procedures

A positive control tube containing 0.1 ng H37Rv DNA and a negative control tube containing no DNA were included with each set of reactions. To evaluate cross-contamination during sampling, we performed control punches using unspotted cards.

Statistical methods

The sensitivity and specificity of each pair of primers for the detection of *M. tuberculosis* were calculated on the basis of the study reference standards, with the liquid culture method taken as a gold standard. In addition, the results of individual PCR were employed for the analysis of smear-positive and -negative samples according to the culture results.

RESULTS

Patient characteristics

We evaluated by PCR 102 sputum samples from 35 TB patients (23 male, 12 female), all of whom were receiving anti-tuberculosis treatment for periods ranging from 2 weeks to 1 year. The average age was 51.4 years.

Gold standard

Twenty-two positive culture samples were taken as gold standard. In all of these samples, *M. tuberculosis* was identified by hybridisation assay.

Appearance of the specimens

Eighty-five per cent of smear positives, 68% of culture positives and 81% of PCR positives contained >30% of purulent sputum (P2 or P3 in Miller & Jones' classification[10]).

Sensitivity detection

Four small discs of the FTA® card system were used as templates for the PCR processing. The criterion for using four discs was based on the assumption that the mycobacteria were scanty and heterogeneously distributed during the absorption process; in addition, in a pilot study we determined that using four disks increased the degree of amplification (data not shown). The detection limit of the PCR assay for the amplification of IS*6110* was 10 fg/μl of purified *M. tuberculosis* H37Rv (Figure 1A). The amplification of the 123 bp fragments by PCR using the FTA® card system is depicted in Figure 1C.

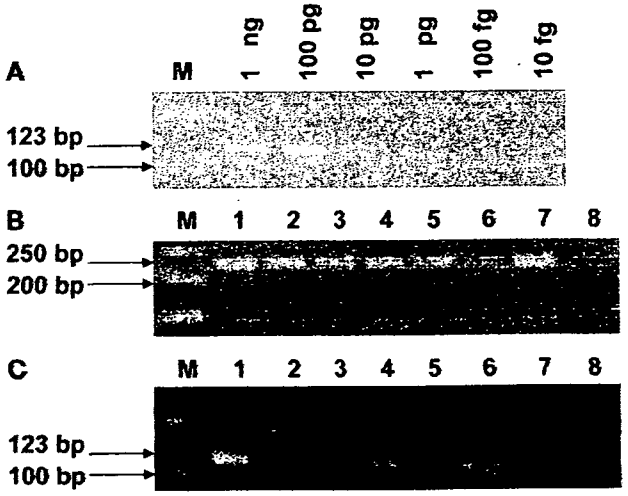

Figure 1 PCR results. **A.** Sensitivity of detection of *M. tuberculosis*: ten-fold serial dilution of H37Rv DNA was amplified. **B.** PCR amplicons from β-globin (250 bp): M: marker; lanes 1–7: results using FTA® cards from TB patients; lane 8: negative control. **C.** PCR amplicons from *M. tuberculosis* (123 bp) stored on FTA® cards: M: marker; lane 1: positive control (H37Rv); lanes 2, 4 and 6: cards from TB patients; lanes 3, 5 and 7: no spotted cards (control punch); lane 8: negative control. In each case, 10 μl samples were electrophoresed through a 1.5% agarose gel and photographed under UV illumination. PCR = polymerase chain reaction; bp = base pairs; TB = tuberculosis; UV = ultraviolet.

Effect of PCR inhibitors

The ability to detect *M. tuberculosis* by PCR can be impaired by the presence of substances inhibitory to Taq DNA polymerase. The β-globin PCR assay generated the expected 250-bp band (Figure 1B) in 90 (89%) of the samples. All the culture-positive samples were also positive in the amplification of β-globin. This finding may suggest that the PCR-negative, culture-positive samples contained low concentrations of TB bacteria rather than PCR inhibitors, which would tend to rule out inhibition as a cause.

PCR and smear results compared with culture

Among the 22 culture-positive samples, 18 (82%) were PCR-positive and 9 (41%) smear-positive (Table 2). The remaining four culture-positive samples were both PCR- and smear-negative. All 9 smear- and culture-positive samples were also positive by PCR. The sensitivity and specificity of PCR were 82% and 96%, compared to 41% and 95%, respectively, for smear examination. Differences were observed on comparing the sensitivity of smear microscopy with that of

Table 2 Comparison of PCR with smear and culture for detection of *M. tuberculosis*

Culture	Positive *n* (%)	Negative *n*
PCR*		
Positive	18 (82)†	3
Negative	4	77
Smear		
Positive	9 (41)†	4
Negative	13	76

* PCR using the FTA® card system.
† (%) sensitivity.
PCR = polymerase chain reaction.

PCR ($P < 0.05$), but there was no observed difference in specificity (Figure 2).

We repeated the PCR procedure for all culture- and smear-positive samples. The second experiment showed the same results as the first.

Sensitivity of PCR by smear result

The sensitivity of PCR for smear-positive, culture-positive samples was 9/9 (100%), whereas that for smear-negative, culture-positive samples was 9/13 (69%). These results show that, even in the paucibacillary form of TB resulting from treatment, this PCR system could provide rapid and sensitive detection of *M. tuberculosis* DNA impregnated on the FTA® card.

Stability and control procedures

All of the 102 samples were analysed by PCR at two time points—at the time of sample collection and after 6 months of storage—obtaining positive amplifications in both cases, clearly showing that storage for 6 months did not affect the amplification. PCR products were not detected in negative controls or control punch cards, confirming the absence of contamination during the procedure (Figure 1C). The total assay time was 9 h.

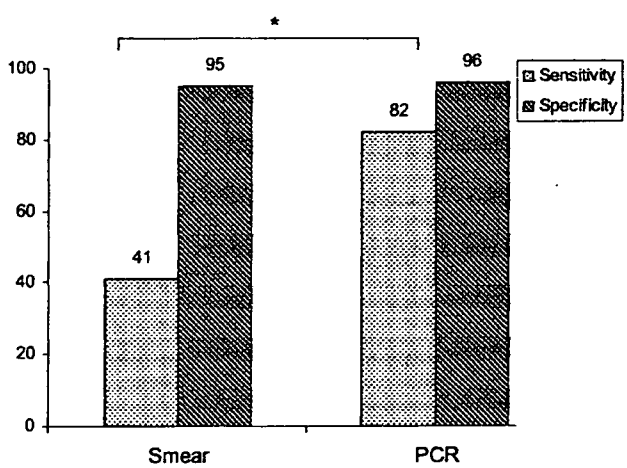

Figure 2 Accuracy of the methods. Sputum smear examination (smear) and PCR assay using the FTA® card system (PCR) among the 22 positive culture samples. * $P < 0.05$. PCR = polymerase chain reaction.

DISCUSSION

The present study demonstrated that the DNA of *M. tuberculosis* can be amplified using sputum spotted on an FTA® card. We found that the *M. tuberculosis* DNA stability with this card at room temperature was up to 6 months. However, care must be taken to avoid cross-contamination between specimens during sampling and handling. The present findings are relevant to patient care and clinical trials and suggest that sputum stored on FTA® cards could provide a simple, economical method for the collection, storage and transport of suspected TB specimens for later testing.

In addition, samples can easily be obtained from geographically isolated populations where access to and/or availability of TB diagnostic testing may be limited. Samples collected may be shipped by mail to a central laboratory for molecular diagnosis without the triple packing system otherwise required for transport.[13]

Depending on the gold standard and other methodological factors, studies have shown PCR sensitivities ranging from 77% to >95% and PCR specificities of >95% in TB patients before treatment.[4,5] Regarding patients under treatment, Kennedy et al. found 76% agreement between culture and PCR.[14] In our study, three culture-negative samples (one smear-positive and two smear-negative) were detected by our method, which may be explained by the limited quantity of TB DNA.[15] While the sensitivity of smear is dependent on the type and quality of the specimen, our method could be employed with accuracy even 6 months after obtaining the sample. However, as we recommend taking the purulent part of the sputum, it will be necessary to help patients understand that secretions from deep in the lung are required.

More sensitive methods exist, including the use of automated culture systems, but the best tests are not always available for the people who most need them.[16]

In summary, the present system appears to be a promising method for transporting and storing sputum samples. Other advantages are the simplicity of the sample preparation and the use of the small disc as a template during the PCR process, using specific targets, without the need for extensive nucleic acid purification.

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RÉSUMÉ

CADRE : La réaction polymérase en chaîne (PCR) est sensible pour la détection de *Mycobacterium tuberculosis*, et est disponible dans la plupart des pays, mais dans une moindre mesure dans les zones rurales.
OBJECTIFS : Amplifier les séquences d'ADN de *M. tuberculosis* des crachats repérées sur les cartes FTA et les comparer avec les résultats des examens microscopiques dans les échantillons de cultures positives.
MÉTHODES : Au total, 102 échantillons de crachats de patients tuberculeux ont été marqués sur les cartes FTA et stockés à l'air ambiant. La spécificité et la sensibilité de deux amorces de PCR qui amplifient la région IS*6110* de *M. tuberculosis* ont été évaluées et comparées à celles de cultures (milieu liquide 7H9), et de l'examen microscopique d'échantillons frais de crachats.
RÉSULTATS : Il a été possible de détecter 10 fg/µl de DNA mycobactérienne même après 6 mois de stockage. L'analyse PCR des deux paires d'amorces révèle une sensibilité et une spécificité respectivement de 82% et 96%, alors que celles de l'examen microscopique sont respectivement de 41% et de 95%.
CONCLUSION : Le système de carte FTA permettant le stockage de l'ADN bactérien issu d'un échantillon de crachats devrait être envisagé pour le diagnostic moléculaire de la tuberculose. Les échantillons de crachats peuvent être facilement obtenus dans des populations géographiquement isolées, stockés et adressés par courrier afin d'établir à distance un diagnostic moléculaire précis.

RESUMEN

CONTEXTO : La reacción en cadena de la polimerasa (PCR) es un método sensible para la detección de *Mycobacterium tuberculosis* y se encuentra al alcance en la mayoría de los países, aunque en menor medida en zonas rurales.
OBJETIVO : Amplificar secuencias del ADN de *M. tuberculosis* a partir de manchas de esputo en papel de filtro (*FTA cards*®) y comparar estos resultados con los resultados de la baciloscopia, en muestras con cultivo positivo para micobacterias.
MÉTODOS : Se recogió un total de 102 muestras de esputo de pacientes con tuberculosis en curso de tratamiento, las cuales se almacenaron como manchas en papel de filtro a temperatura ambiente hasta el momento del análisis. Con la PCR se amplificó un fragmento de 123 pares de bases de la secuencia de inserción IS*6110* de *M. tuberculosis*. Se evaluó la eficacia de la PCR en la detección de *M. tuberculosis* y los resultados se compararon con los resultados de los cultivos en medio líquido 7H9 (método de referencia) y de la baciloscopia, de muestras frescas de esputo.
RESULTADOS : El método permitió detectar hasta 10 fg/µl de ADN micobacteriano en muestras almacenadas durante más de 6 meses. La PCR a partir de las manchas de esputo en tarjetas FTA® mostró una sensibilidad del 82% y una especificidad del 96%, comparada con una sensibilidad del 41% y una especificidad del 95% de la baciloscopia.
CONCLUSIÓN : El sistema con tarjetas FTA® debería tenerse en cuenta como método de conservación del ADN micobacteriano presente en las muestras de esputo, para el diagnóstico molecular de la tuberculosis. Así, en poblaciones geográficamente aisladas, de manera sencilla podrían obtenerse las muestras de esputo, almacenarlas y expedirlas por correo con el fin de establecer un diagnóstico molecular exacto.

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SHORT COMMUNICATION

Low antibody response against tuberculous glycolipid (TBGL) in elderly gastrectomised tuberculosis patients

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SUMMARY

To evaluate differences in anti-tuberculous glycolipid (TBGL) antibody titers in patients who developed tuberculosis (TB) with and without gastrectomy, 11 gastrectomised patients who developed TB after surgery (GS-TB), 19 TB patients without any other complications (TB), 12 gastrectomised patients who did not develop TB after surgery (GS) and 27 healthy subjects (H) with normal findings on chest X-ray were evaluated, although there were no differences in the clinical findings at admission between the TB and GS-TB groups. The assay used here allowed us to find low anti-TBGL antibody titers in GS-TB patients.

KEY WORDS: TBGL; gastrectomy; tuberculosis

GASTRECTOMY is known as an associated co-factor in the development of tuberculosis (TB). Although the reasons for the association have not been clarified,[1,2] previous studies with a significant number of gastrectomised patients have reported prevalences of 1.7% to 2.5%. In addition, immunodeficiency and/or malnutrition contribute to the development of TB.[3] We therefore evaluated the clinical and laboratory findings, including purified protein derivative (PPD) reaction, in TB and/or gastrectomised patients and healthy controls. In the gastrectomised patients, group 2 lymph nodes were dissected, which could have affected the host immune responses. The glycolipid antigen trehalose 6, 6′-dimycolate (TDM) purified from *Mycobacterium tuberculosis* H37Rv has recently been reported as a useful diagnostic antigen.[4–6] We thus investigated specific immune responses against TDM by measuring anti-tuberculous glycolipid (TBGL) antibody titers.

Between 1999 and 2001, a total of 60 patients from Tohoku University Hospital enrolled in the study were divided into four groups: 1) gastrectomised patients who developed TB after surgery (GS-TB, n = 11); 2) TB patients without any other complications (TB, n = 19); 3) gastrectomised patients who did not develop TB after surgery (GS, n = 12); and 4) healthy subjects (n = 27) with normal findings on chest X-ray. For both TB and GS-TB patients, blood samples were taken before anti-tuberculosis drugs were given.

The study was approved by the ethics committee of the Tohoku University School of Medicine. Informed consent was obtained from patients and volunteers to participate in the study. For the diagnosis of TB, in addition to clinical features, acid-fast smear, culture and polymerase chain reaction tests (Roche Amplicor Mycobacterium Kit, Branchburg, NJ, USA) were performed using sputum or gastric fluid. Gastrectomised patients with recurring cancer, those treated with anticancer drugs and those with other diseases were excluded. Immunoglobulin G (IgG) antibodies against TBGL antigen was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Kyowa Medex Co, Tokyo, Japan). A cut-off value ≥2 U/ml was considered positive.[4,5] Statistical analyses were performed using conventional methods.

Mean age and postoperative duration were 74.5 ± 9.2 years and 24.3 ± 6.3 months in the GS-TB group and 74.8 ± 3.0 years and 20.6 ± 9.2 months in the GS group, respectively. There were no significant differences between the two groups ($P > 0.05$). The mean ages in the TB patients and healthy controls were respectively 73.0 ± 9.4 and 73.2 ± 8.2 years. There were no significant differences in positive tuberculin test (6/11 vs. 10/19), lung infiltration shadow (≥2 lobes) (5/11 vs. 14/19) or acid-fast smear (≥1/field) (6/11 vs. 10/19) between the GS-TB and TB groups.

In this study, 55% of the GS-TB patients were positive for the anti-TBGL antibody titers by ELISA; this result was comparable to that obtained for the TB patients (68%). Maekura et al. found a significant number (17%) of TBGL-positive serum samples in healthy individuals.[5] In our healthy subjects, 22% of serum samples showed positive responses, but curiously none of the GS group was positive. Furthermore, the anti-TBGL antibody titers in the GS-TB patients were

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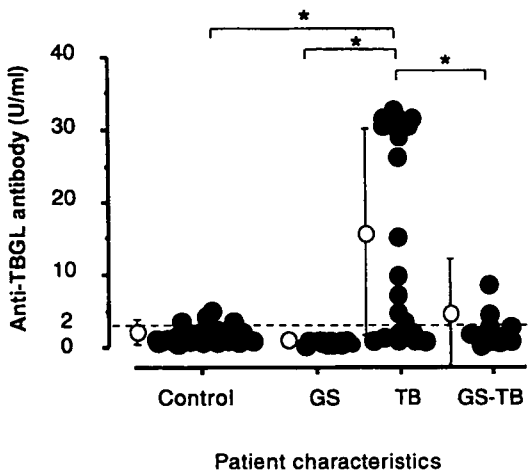

Figure 1 Individual anti-TBGL values in healthy controls and GS, TB and GS-TB patients. Circles = individual anti-TBGL values; bars = standard deviation; dotted line = cut-off; anti-TBGL = anti-tuberculous glycolipid; GS = gastrectomised; TB = tuberculosis. * $P < 0.05$.

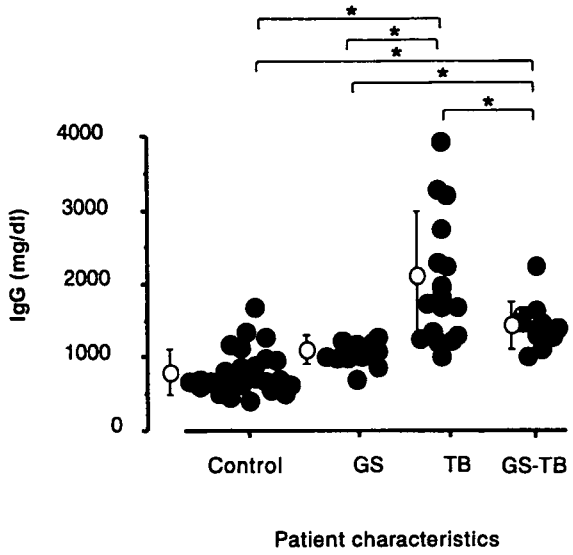

Figure 2 Individual IgG values in healthy controls and GS, TB and GS-TB patients. Circles = individual IgG values; bars = standard deviation; IgG = immunoglobulin G; GS = gastrectomised; TB = tuberculosis. * $P < 0.05$.

significantly lower than those of the TB patients ($P < 0.05$) (Figure 1). The serum IgG in both TB and GS-TB patients was significantly higher than in the GS patients and healthy controls (Figure 2), confirming that the decrease of anti-TBGL antibody in GS-TB patients could not be explained by the low IgG in their serum. It should also be noted that both anti-TBGL antibody and total IgG values were lower among GS-TB patients than in the TB group.

The reason for the lack of anti-TBGL antibody in GS patients and the low anti-TBGL antibody titers in GS-TB patients is not clear, but both suggest that the stomach may play a role in the production of anti-TBGL antibodies.

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RÉSUMÉ

Afin d'évaluer les différences entre les titres d'anticorps glycolipides anti-tuberculeux (TBGL) chez des patients ayant développé TB avec ou sans gastrectomie, ont été évalués 11 patients gastrectomisés ayant développé une TB après chirurgie (GS-TB), 19 patients TB sans autre complication (TB), 12 patients gastrectomisés n'ayant pas développé de TB après chirurgie (GS), et 27 sujets sains (H) présentant des radiographies thoraciques normales, bien qu'il n'y a eu aucune différence dans les investigations cliniques à l'admission entre les groupes TB et GS-TB. L'essai utilisé nous a permis de trouver de faibles titres d'anticorps d'anti-TBGL chez les patients GS-TB.

RESUMEN

Para evaluar la diferencia de títulos de anticuerpos para un glicolípido antituberculoso (TBGL), en pacientes infectados con TB con y sin antecedente de gastrectomía, se evaluaron 11 pacientes gastrectomisados (GS-TB), 19 pacientes infectados con TB sin otra complicación (TB), 12 pacientes gastrectomisados que no desarrollaron TB (GS) y 27 sanos (H) con exámenes radiológicos normales. No hubo diferencias significativas entre los grupos TB y GS-TB al ingreso. Los resultados mostraron bajos títulos anti-TBGL en pacientes GS-TB.

Enhanced TLR-mediated NF-IL6–dependent gene expression by Trib1 deficiency

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Toll-like receptors (TLRs) recognize a variety of microbial components and mediate downstream signal transduction pathways that culminate in the activation of nuclear factor κB (NF-κB) and mitogen-activated protein (MAP) kinases. Trib1 is reportedly involved in the regulation of NF-κB and MAP kinases, as well as gene expression in vitro. To clarify the physiological function of Trib1 in TLR-mediated responses, we generated Trib1-deficient mice by gene targeting. Microarray analysis showed that Trib1-deficient macrophages exhibited a dysregulated expression pattern of lipopolysaccharide-inducible genes, whereas TLR-mediated activation of MAP kinases and NF-κB was normal. Trib1 was found to associate with NF-IL6 (also known as CCAAT/enhancer-binding protein β). NF-IL6–deficient cells showed opposite phenotypes to those in Trib1-deficient cells in terms of TLR-mediated responses. Moreover, overexpression of Trib1 inhibited NF-IL6–dependent gene expression by down-regulating NF-IL6 protein expression. In contrast, Trib1-deficient cells exhibited augmented NF-IL6 DNA-binding activities with increased amounts of NF-IL6 proteins. These results demonstrate that Trib1 is a negative regulator of NF-IL6 protein expression and modulates NF-IL6–dependent gene expression in TLR-mediated signaling.

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Abbreviations used: 24p3, lipocalin-2; BLP, bacterial lipoprotein; C/EBP, CCAAT/enhancer-binding protein; Jnk, c-Jun N-terminal kinase; MALP-2, macrophage-activating lipopeptide–2; MAP, mitogen-activated protein; mPGES, prostaglandin E synthase; TLR, Toll-like receptor.

Innate immunity is promptly activated after the invasion of microbes through recognition of pathogen-associated molecular patterns by pattern-recognition receptors, including Toll-like receptors (TLRs) (1). The recognition of microbial components by TLRs effectively stimulates host immune responses such as proinflammatory cytokine production, cellular proliferation, and up-regulation of co-stimulatory molecules, accompanied by the activation of NF-κB and mitogen-activated protein (MAP) kinases (2, 3). Although the inhibitory protein IκB family members sequester NF-κB in the cytoplasm of unstimulated cells, TLR-dependent IκB phosphorylation by the IκB kinase complex and degradation by the ubiquitin–proteasome pathway permit translocation of NF-κB to the nucleus (4). MAP kinases such as c-Jun N-terminal kinase (Jnk) and p38 are also rapidly phosphorylated and activated by upstream kinases in response to TLR stimulation (5). Moreover, TLR-mediated activity of NF-κB and MAP kinases is shown to be regulated at multiple steps regarding the strength and the duration of the activation (6).

Recent extensive experiments have identified a variety of modulators that have positive and negative effects on the activation of NF-κB and MAP kinases, including a family of serine/threonine kinase-like proteins called Trib (7). Trib consists of three family members: Trib1 (also known as c8fw, GIG2, or SKIP1), Trib2 (also known as c5fw), and Trib3 (also known as NIPK, SINK, or SKIP3) (7–12). Trib3 has been shown to interact with the p65 subunit of NF-κB and to inhibit NF-κB–dependent gene expression in vitro (11). In terms of MAP kinases, Trib1, Trib2, and Trib3 reportedly bind to Jnk and p38, and affect the activity of MAP kinases and IL-8 production in response to PMA or

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TLR ligands/IL-1 (12). However, whether Trib family members regulate TLR-mediated signaling pathways under physiological conditions is still unknown.

In this study, we generated Trib1-deficient mice by gene targeting and analyzed TLR-mediated responses. Although the activation of NF-κB and MAP kinases in response to LPS was comparable between wild-type and Trib1-deficient cells, microarray analysis revealed that a subset of LPS-inducible genes was dysregulated in Trib1-deficient cells. Subsequent yeast two-hybrid analysis identified the CCAAT/enhancer-binding protein (C/EBP) family member NF-IL6 (also known as C/EBPβ) as a binding partner of Trib1, and phenotypes found in NF-IL6–deficient cells were opposite to those observed in Trib1-deficient cells. Moreover, overexpression of Trib1 inhibited NF-IL6–mediated gene expression and reduced amounts of NF-IL6 proteins. Inversely, NF-IL6 DNA-binding activity and LPS-inducible NF-IL6–target gene expression were up-regulated in Trib1-deficient cells, in which amounts of NF-IL6 proteins were increased. These results demonstrate that Trib1 plays an important role in NF-IL6–dependent gene expression in the TLR-mediated signaling pathways.

RESULTS

Comprehensive gene expression analysis in Trib1-deficient macrophages

To assess the physiological function of Trib1 in TLR-mediated immune responses, we performed a microarray analysis to compare gene expression profiles between wild-type and Trib1-deficient macrophages in response to LPS (Fig. 1 A and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20070183/DC1). Out of 45,102 transcripts, we first defined the genes induced more than twofold after LPS stimulation in wild-type cells as "LPS-inducible genes" and identified 790 of them (Table S1). We next compared the LPS-inducible genes in wild-type and Trib1-deficient macrophages after LPS stimulation and found 59, 703, and 28 genes as up-regulated, similarly expressed, and down-regulated in Trib1-deficient cells, respectively (Table S1).

Among the up-regulated genes, several were subsequently tested by Northern blotting to confirm the accuracy. LPS-induced expression of prostaglandin E synthase (mPGES), lipocalin-2 (24p3), arginase type II, and plasminogen activator inhibitor type II, which were highly up-regulated in the microarray analysis (Table S1), was indeed enhanced in Trib1-deficient macrophages (Fig. 1 B). Furthermore, in contrast to proinflammatory cytokines such as TNF-α and IL-6, which were similarly expressed between wild-type and Trib1-deficient cells in response not only to LPS but also to other TLR ligands, IL-12 p40 was down-regulated in Trib1-deficient cells compared with wild-type cells (Fig. 1 C; Fig. S2, A–C, available at http://www.jem.org/cgi/content/full/jem.20070183/DC1; and Table S1). Thus, the comprehensive microarray analysis revealed that a subset of LPS-inducible genes is dysregulated in Trib1-deficient cells.

Previous in vitro studies demonstrate that human Trib family members modulate activation of MAP kinases and

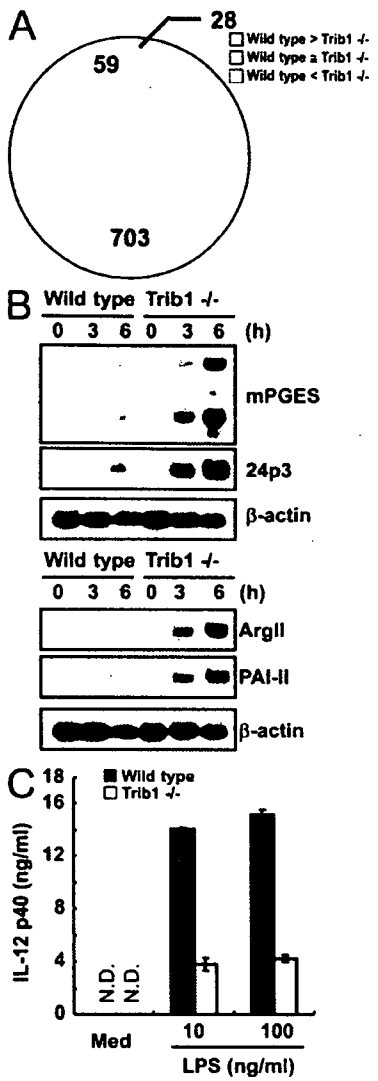

Figure 1. Dysregulation of a subset of LPS-inducible genes in Trib1-deficient cells. (A) Summary of DNA chip microarray analysis. 790 LPS-inducible genes were divided into up-regulated (yellow), similarly expressed (pink), and down-regulated (blue) groups, with the indicated amounts of each. (B) Peritoneal macrophages from wild-type or Trib1-deficient mice were stimulated with 10 ng/ml LPS for the indicated periods. Total RNA (10 μg) was extracted and subjected to Northern blot analysis for the expression of the indicated probes. (C) Peritoneal macrophages from wild-type and Trib1-deficient mice were cultured with the indicated concentrations of LPS in the presence of 30 ng/ml IFN-γ for 24 h. Concentrations of IL-12 p40 in the culture supernatants were measured by ELISA. Indicated values are means ± SD of triplicates. Data are representative of three (B) or two (C) independent experiments. N.D., not detected.

NF-κB (7–12). Both wild-type and Trib1-deficient cells showed similar levels and time courses of phosphorylation of p38, Jnk and extracellular signal-regulated kinase, and IκBα degradation (Fig. S2 D), indicating that the dysregulated

expression of LPS-inducible genes in Trib1-deficient cells might be the independent of activation of NF-κB and MAP kinases.

Interaction of Trib1 with NF-IL6

To explore signaling aspects of Trib1 deficiency other than NF-κB and MAP kinases, we performed a yeast-two-hybrid screen with the full length of human Trib1 as bait to identify a binding partner of Trib1 and identified several clones as being positive. Sequence analysis subsequently revealed that three clones encoded the N-terminal portion of a member of the C/EBP NF-IL6 (unpublished data). We initially tested the interaction of Trib1 and NF-IL6 in yeasts. AH109 cells were transformed with a plasmid encoding the full length of Trib1 together with a plasmid encoding the N-terminal portion of NF-IL6 obtained by the screening (Fig. 2 A). We next examined the interaction in mammalian cells using immunoprecipitation experiments. HEK293 cells were transiently transfected with a plasmid encoding the full length of mouse Trib1 together with a plasmid encoding the full length of mouse NF-IL6. Myc-tagged NF-IL6 was coimmunoprecipitated with Flag-Trib1 (Fig. 2 B), showing the interaction of Trib1 and NF-IL6 in mammalian cells.

TLR-mediated immune responses in NF-IL6–deficient macrophages

An in vitro study showing the interaction of Trib1 and NF-IL6 prompted us to examine the TLR-mediated immune responses in NF-IL6–deficient cells, because LPS-induced expression of mPGES is shown to depend on NF-IL6 (13). We initially analyzed the expression pattern of genes affected by the loss of Trib1 in NF-IL6–deficient macrophages by Northern blotting. LPS-induced expression of 24p3, plasminogen activator inhibitor type II, and arginase type II, as well as mPGES, was profoundly defective in NF-IL6–deficient cells (Fig. 2 C). We next tested IL-12 p40 production by ELISA. As previously reported, IL-12 p40 production by LPS stimulation was increased in a dose-dependent fashion in NF-IL6–deficient cells compared with control cells (Fig. 2 D) (14). In addition, the production in response to bacterial lipoprotein (BLP), macrophage-activating lipopeptide–2 (MALP-2), or CpG DNA was also augmented in

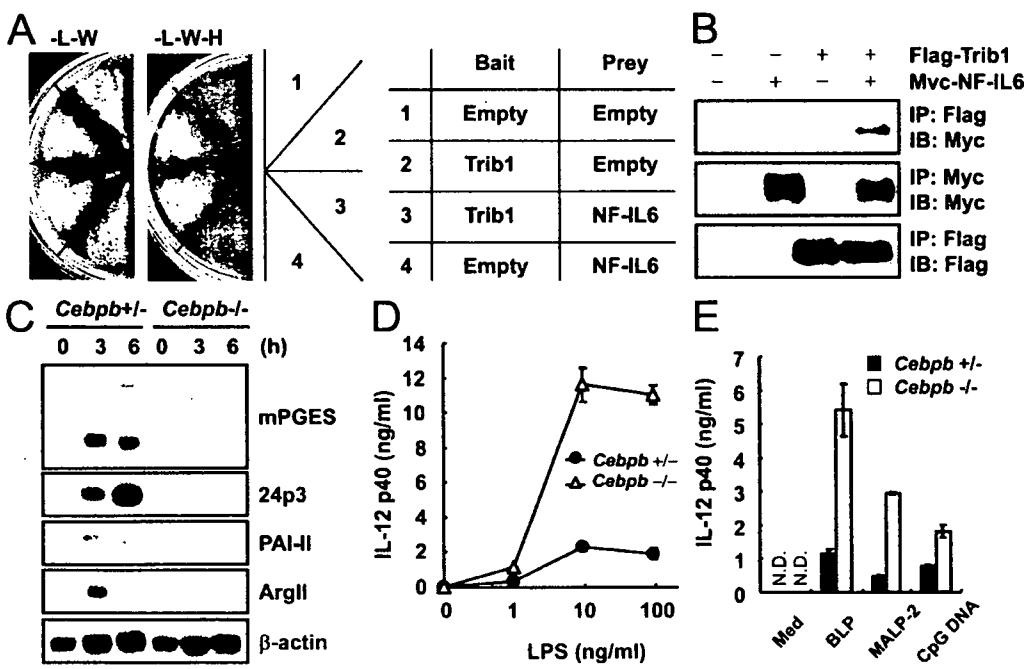

Figure 2. Association of Trib1 with NF-IL6 and TLR-mediated responses in NF-IL6–deficient macrophages. (A) Plasmids expressing human Trib1 fused to the GAL4 DNA-binding domain or an empty vector were cotransfected with a plasmid expressing NF-IL6 fused to GAL4 transactivation domain or an empty vector. Interactions were detected by the ability of cells to grow on medium lacking tryptophan, leucin, and histidine (-L-W-H). The growth of cells on a plate lacking tryptophan and leucine (-L-W) is indicative of the efficiency of the transfection. (B) Lysates of HEK293 cells transiently cotransfected with 2 μg of Flag-tagged Trib1 and/or 2 μg Myc-tagged NF-IL6 expression vectors were immunoprecipitated with the indicated antibodies. (C) Peritoneal macrophages from wild-type or NF-IL6–deficient mice were stimulated with 10 ng/ml LPS for the indicated periods. Total RNA (10 μg) was extracted and subjected to Northern blot analysis for expression of the indicated probes. (D and E) Peritoneal macrophages from wild-type and NF-IL6–deficient mice were cultured with the indicated concentrations of LPS (D) or with 100 ng/ml BLP, 30 ng/ml MALP-2, or 1 μM, CpG DNA (E) in the presence of 30 ng/ml IFN-γ for 24 h. Concentrations of IL-12 p40 in the culture supernatants were measured by ELISA. Indicated values are means ± SD of triplicates. Data are representative of three (B) and two (C–E) separate experiments. N.D., not detected.

NF-IL6–deficient cells (Fig. 2 E). Together, compared with Trib1-deficient cells, converse phenotypes in terms of TLR-mediated immune responses are observed in NF-IL6–deficient cells.

Inhibition of NF-IL6 by Trib1 overexpression

To test whether Trib1 down-regulates NF-IL6–dependent activation, HEK293 cells were transfected with an NF-IL6–dependent luciferase reporter plasmid together with NF-IL6 and various amounts of Trib1 expression vectors (Fig. 3 A). NF-IL6–mediated luciferase activity was diminished by co-expression of Trib1 in a dose-dependent manner. Moreover, RAW264.7 macrophage cells overexpressing Trib1 exhibited reduced expression of mPGES and 24p3 in response to LPS (Fig. S3 A, available at http://www.jem.org/cgi/content/full/jem.20070183/DC1). We next tested NF-IL6 DNA-binding activity by EMSA and observed less NF-IL6 DNA-binding activity in HEK293 cells coexpressing NF-IL6 and Trib1 than in ones transfected with the NF-IL6 vector alone (Fig. 3 B), presumably accounting for the down-regulation of the NF-IL6–dependent gene expression by Trib1. We then examined the effect of Trib1 on the amounts of NF-IL6 proteins by Western blotting. Although the diminution of NF-IL6 by Trib1 was marginal when excess amounts of NF-IL6 were expressed, we found that the transient expression of lower levels of NF-IL6, together with Trib1, resulted in a reduction of NF-IL6 in HEK293 cells (Fig. 3 C). Also, endogenous levels of NF-IL6 proteins in RAW264.7 cells overexpressing Trib1 were markedly less than those in control cells (Fig. 3 D). These results demonstrated that overproduction of Trib1 might negatively regulate NF-IL6 activity in vitro.

Up-regulation of NF-IL6 in Trib1-deficient cells

We next attempted to check the in vivo status of NF-IL6 in Trib1-deficient cells by comparing the NF-IL6 DNA-binding activity in Trib1-deficient macrophages with that in wild-type cells by EMSA. Although LPS-induced NF-κB–DNA complex formation in Trib1-deficient cells was similarly observed, Trib1-deficient cells exhibited elevated levels of C/EBP–DNA complex formation compared with wild-type cells (Fig. 4 A). We further examined whether the C/EBP–DNA complex in Trib1-deficient cells contained NF-IL6 by supershift assay. Addition of anti–NF-IL6 antibody into the C/EBP–DNA complex yielded more supershifted bands in Trib1-deficient cells than in wild-type cells (Fig. 4 B). In addition, the C/EBP–DNA complex was not shifted by the addition of anti-C/EBPδ (also known as NF-IL6β) antibody (Fig. S4 A, available at http://www.jem.org/cgi/content/full/jem.20070183/DC1), suggesting that NF-IL6 DNA-binding activity is augmented in Trib1-deficient cells. We then examined the amounts of NF-IL6 proteins by Western blotting (Fig. 4 C). Compared with wild-type cells, Trib1-deficient cells showed increased levels of NF-IL6 proteins. Finally, we examined NF-IL6 mRNA levels by Northern blotting and observed enhanced expression of NF-IL6 mRNA in Trib1-deficient cells (Fig. 4 D), which is consistent with the autocrine induction of NF-IL6 mRNA

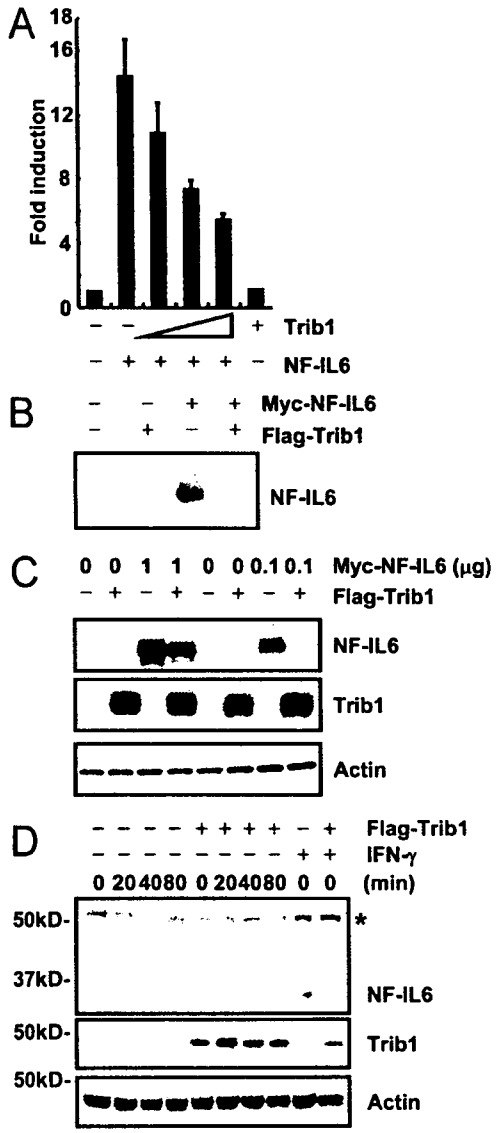

Figure 3. Inhibition of NF-IL6 activity by Trib1 overexpression. (A) HEK293 cells were transfected with an NF-IL6–dependent luciferase reporter together with either Trib1 and/or NF-IL6 expression plasmids. Luciferase activities were expressed as the fold increase over the background shown by lysates prepared from mock-transfected cells. Indicated values are means ± SD of triplicates. (B) HEK293 cells were transfected with 0.1 μg NF-IL6 expression vector together with 4 μg Trib1 expression plasmids. Nuclear extracts were prepared, and C/EBP DNA-binding activity was determined by EMSA using a probe containing the NF-IL6 binding sequence from the mouse 24p3 gene. (C) Lysates of HEK293 cells transiently cotransfected with 2 μg of Flag-tagged Trib1 alone or the indicated amounts of Myc-tagged NF-IL6 expression vectors were immunoblotted with anti-Myc or -Flag for detection of NF-IL6 or Trib1, respectively. (E) RAW 264.7 cells stably transfected with either an empty vector or Flag-Trib1 were stimulated with 10 ng/ml LPS for the indicated periods. The cell lysates were immunoblotted with the indicated antibodies. A protein that cross-reacts with the antibody is indicated (*). Data are representative of three (A and C) and two (B and D), separate experiments.

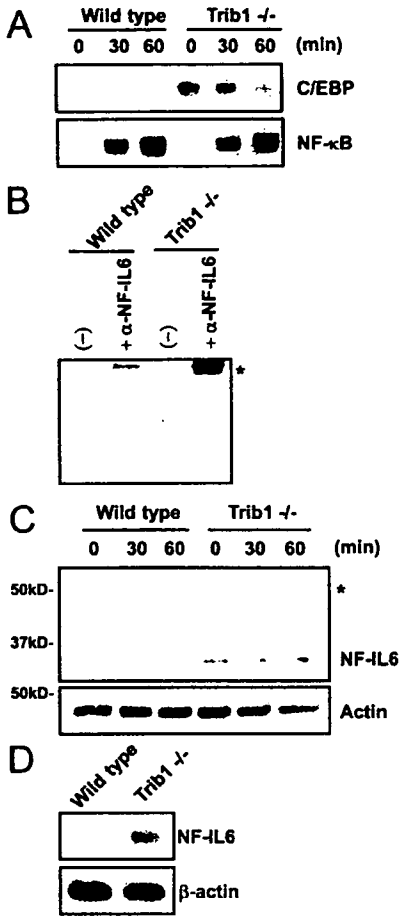

Figure 4. Up-regulation of NF-IL6 activity in Trib1-deficient cells. (A) Peritoneal macrophages from wild-type or Trib1-deficient mice were stimulated with 10 ng/ml LPS for the indicated periods. Nuclear extracts were prepared, and C/EBP DNA-binding activity was determined by EMSA using a C/EBP consensus probe. (B) Nuclear extracts of wild-type and Trib1-deficient unstimulated macrophages were preincubated with anti-NF-IL6, followed by EMSA to determine the C/EBP DNA-binding activity. Supershifted bands are indicated (*). (C) Peritoneal macrophages from wild-type or Trib1-deficient mice were stimulated with 10 ng/ml LPS for the indicated periods and lysed. The cell lysates were immunoblotted with the indicated antibodies. A protein that cross-reacts with the antibody is indicated (*). (D) Total RNA (10 μg) from unstimulated peritoneal macrophages from wild-type or NF-IL6-deficient mice was extracted and subjected to Northern blot analysis for expression of the indicated probes. Data are representative of two (A and B) and three (C and D) separate experiments.

in a previous study (15). Thus, Trib1 may negatively control amounts of NF-IL6 proteins, thereby affecting TLR-mediated NF-IL6–dependent gene induction.

DISCUSSION

In this study, we demonstrate by microarray analysis and biochemical studies that Trib1 is associated with NF-IL6 and negates NF-IL6–dependent gene expression by reducing the amounts of NF-IL6 proteins in the context of TLR-mediated responses. Especially regarding IL-12 p40, although the microarray data showed an almost twofold reduction of the mRNA in Trib1-deficient cells (Table S1), the production was three to four times lower than that in wild-type cells (Fig. 1 C), suggesting translational control of IL-12 p40 by Trib1 in addition to the transcriptional regulation. Moreover, the transcription of the IL-12 p40 gene itself may be affected by not only the amount of NF-IL6 proteins but also the phosphorylation or the isoforms such as liver-enriched activator protein and liver-enriched inhibitory protein (16–18). The molecular mechanisms of how Trib1 deficiency affects IL-12 p40 production on the transcriptional or translational levels through NF-IL6 regulation need to be carefully studied in the future.

The name Trib is originally derived from the *Drosophila* mutant strain *tribbles*, in which the *Drosophila* tribbles protein negatively regulates the level of *Drosophila* C/EBP *slbo* protein and C/EBP-dependent developmental responses such as border cell migration in larvae (19–22). It is also of interest that Trib1-deficient female mice and *Drosophila* in adulthood are both infertile (unpublished data) (18). In mammals, other Trib family members such as Trib2 and Trib3 have recently been shown to be involved in C/EBP-dependent responses (23, 24). Mice transferred with bone marrow cells, in which Trib2 is retrovirally overexpressed, display acute myelogenous leukemia–like disease with reduced activities and amounts of C/EBPα (23). In addition, ectopic expression of Trib3 inhibits C/EBP-homologous protein–induced ER stress–mediated apoptosis (24). Thus, the function of tribbles to inhibit C/EBP activities by controlling the amounts appears to be conserved throughout evolution.

Given the up-regulation of the mRNA in Trib1-deficient cells (Fig. 4 D), the reduction of NF-IL6 in Trib1-overexpressing cells (Fig. 3 C), the auto-regulation of NF-IL6 by itself (15), and the degradation of C/EBPα by Trib2 (23) and slbo by tribbles (22), the loss of Trib1 might primarily result in impaired degradation of NF-IL6 and, subsequently, in excessive accumulation of NF-IL6 via the autoregulation in Trib1-deficient cells.

In this study, we focused on the involvement of Trib1 in TLR-mediated NF-IL6–dependent gene expression. However, given that the levels of NF-IL6 proteins were increased in Trib1-deficient cells, it is reasonable to propose that other non–TLR-related NF-IL6–dependent responses might be enhanced in Trib1-deficient mice. Moreover, Trib3 is also shown to be involved in insulin-mediated Akt/PKB activation in the liver by mechanisms apparently unrelated to C/EBP, suggesting that Trib family members possibly function in a C/EBP-independent fashion (25–27). Future studies using mice lacking other Trib family members, as well as Trib1, may help to unravel the nature of mammalian tribbles in wider points of view.

MATERIALS AND METHODS

Generation of Trib1-deficient mice. A genomic DNA containing the *Trib1* gene was isolated from the 129/SV mouse genomic library and characterized by restriction enzyme mapping and sequencing analysis. The gene encoding mouse Trib1 consists of three exons. The targeting vector was constructed by replacing a 0.4-kb fragment encoding the second exon of the

Trib1 gene with a neomycin resistance gene cassette (*neo*) (Fig. S1 A). The targeting vector was transfected into embryonic stem cells (E14.1). G418 and ganeyclovir doubly resistant colonies were selected and screened by PCR and Southern blot analysis (Fig. S1 B). Homologous recombinants were microinjected into C57BL/6 female mice, and heterozygous F1 progenies were intercrossed to obtain *Trib1*$^{-/-}$ mice. We interbred the heterozygous mice to produce offspring carrying a null mutation of the gene encoding Trib1. Trib1-deficient mice were born at the expected Mendelian ratio and showed a slight growth retardation with reduced body weight until 2–3 wk after birth (unpublished data). Trib1-deficient that mice survived for >6 wk were analyzed in this study. To confirm the disruption of the gene encoding Trib1, we analyzed total RNA from wild-type and Trib1-deficient peritoneal macrophages by Northern blotting and found no transcripts for Trib1 in Trib1-deficient cells (Fig. S1 C). All animal experiments were conducted with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases at Osaka University.

Reagents, cells, and mice. LPS (a TLR4 ligand) from *Salmonella minnesota* Re 595 and anti-Flag were purchased from Sigma-Aldrich. BLP (TLR1/TLR2), MALP-2 (TLR2/TLR6), and CpG oligodeoxynucleotides (TLR9) were prepared as previously described (28). Antiphosphorylated extracellular signal-regulated kinase, Jnk, and p38 antibodies were purchased from Cell Signaling. Anti–NF-IL6 (C/EBPβ), C/EBPδ, actin, IκBα, and Myc-probe were obtained from Santa Cruz Biotechnology, Inc. NF-IL6–deficient mice were as previously described (29). Epitope-tagged Trib1 fragments were generated by PCR using cDNA from LPS-stimulated mouse peritoneal macrophages as the template and cloned into pcDNA3 expression vectors, according to the manufacturer's instructions (Invitrogen).

Measurement of proinflammatory cytokine concentrations. Peritoneal macrophages were collected from peritoneal cavities 96 h after thioglycollate injection and cultured in 96-well plates (10^5 cells per well) with the indicated concentrations of the indicated ligands for 24 h, as shown in the figures. Concentrations of TNF-α, IL-6, and IL-12 p40 in the culture supernatant were measured by ELISA, according to manufacturer's instructions (TNF-α and IL-12 p40, Genzyme; IL-6, R&D Systems).

Luciferase reporter assay. The NF-IL6–dependent reporter plasmids were constructed by inserting the promoter regions (−1200 to +53) of the mouse 24p3 gene amplified by PCR into the pGL3 reporter plasmid. The reporter plasmids were transiently cotransfected into HEK293 with the control *Renilla* luciferase expression vectors using a reagent (Lipofectamine 2000; Invitrogen). Luciferase activities of total cell lysates were measured using the Dual-Luciferase Reporter Assay System (Promega), as previously described (28).

Yeast two-hybrid analysis. Yeast two-hybrid screening was performed as described for the Matchmaker two-hybrid system 3 (CLONTECH Laboratories, Inc.). For construction of the bait plasmid, the full length of human Trib1 was cloned in frame into the GAL4 DNA-binding domain of pGBKT7. Yeast strain AH109 was transformed with the bait plasmid plus the human lung Matchmaker cDNA library. After screening of 10^6 clones, positive clones were picked, and the pACT2 library plasmids were recovered from individual clones and expanded in *Escherichia coli*. The insert cDNA was sequenced and characterized with the BLAST program (National Center for Biotechnology Information).

Microarray analysis. Peritoneal macrophages from wild-type or Trib1-deficient mice were left untreated or were treated for 4 h with 10 ng/ml LPS in the presence of 30 ng/ml IFN-γ. The cDNA was synthesized and hybridized to Murine Genome 430 2.0 microarray chips (Affymetrix), according to the manufacturer's instructions. Hybridized chips were stained and washed and were scanned with a scanner (GeneArray; Affymetrix). Microarray Suite software (version 5.0; Affymetrix) was used for data analysis. Microarray data have been deposited in the Gene Expression Omnibus under accession no. GSE8788.

Western blot analysis and immunoprecipitation. Peritoneal macrophages were stimulated with the indicated ligands for the indicated periods, as shown in the figures. The cells were lysed in a lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-Cl [pH 7.5], 5 mM EDTA) and a protease inhibitor cocktail (Roche). The cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. For immunoprecipitation, cell lysates were precleared with protein G–sepharose (GE Healthcare) for 2 h and incubated with protein G–sepharose containing 1 μg of the antibodies indicated in the figures for 12 h, with rotation at 4°C. The immunoprecipitants were washed four times with lysis buffer, eluted by boiling with Laemmli sample buffer, and subjected to Western blot analysis using the indicated antibodies, as previously described (28).

EMSA and supershift assay. 2 × 10^6 peritoneal macrophages were stimulated with the indicated stimulants for the indicated periods, as shown in the figures. 2 × 10^6 HEK293 cells were transfected with 0.1 μg Myc–NF-IL6 and/or 4 μg Flag-Trib1 expression vectors. Nuclear extracts were purified from cells and incubated with a probe containing a consensus C/EBP DNA-binding sequence (5′-TGCAGATTGCGCAATCTGCA-3′; Fig. 4, A and B) or mouse 24p3 NF-IL6 binding sequence (sense, 5′-CTTCCTGTTGCTCAACCTTGCA-3′;antisense,5′-TGCAAGGTTGAGCAACAGGAAG-3′; Fig. 3 B), electrophoresed, and visualized by autoradiography, as previously described (28, 30). When the supershift assay was performed, nuclear extracts were mixed with the supershift-grade antibodies indicated in the figures before the incubation with the probes for 1 h on ice.

Online supplemental material. Fig. S1 showed our strategy for the targeted disruption of the mouse *Trib1* gene. Fig. S2 showed the status of proinflammatory cytokine production in response to various TLR ligands and LPS-induced activation of MAP kinases and IκB degradation. Fig. S3 showed decreased expression of NF-IL6–dependent gene in Trib1-overexpressing cells. Fig. S4 showed that the C/EBP-DNA complex in Trib1-deficient cells contained NF-IL6, but not C/EBPδ. Table S1 provides a complete list of the LPS-inducible genes studied. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20070183/DC1.

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第82回総会シンポジウム

Ⅱ. 抗酸菌検査法

座長 [1]高嶋 哲也 [2]樋口 武史

キーワーズ：迅速検査，精度保証，採痰指導，同定検査，遺伝子検査，感受性検査

シンポジスト：

1. わが国における抗酸菌検査の現状と精度保証
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2000年に本学会抗酸菌検査法検討委員会は，結核菌検査診断技術の目覚ましい進歩と普及に鑑みて，「新結核菌検査指針2000」を刊行した。その後も相次いで，感度・特異度ならびに迅速性の向上を目指した検査法が開発・導入されている。そこで本委員会は，7年ぶりに結核菌検査指針の改訂を行い，現状に即した内容に整理することになった。「結核菌検査指針2007」は，塗抹検査を24時間以内に，結核菌の分離・同定を21日以内に，そして薬剤感受性結果を30日以内に報告することを求めたCDC勧告に沿った迅速検査体制の構築と抗酸菌検査の精度管理の普及に主眼をおいて執筆された。本シンポジウムは，この「結核菌検査指針2007」の改訂の主旨を広く知っていただくことを目的に企画した。

迅速検査体制の柱となる液体培養法や，液体培地を用いた薬剤感受性検査あるいは遺伝子検査などがどの程度まで普及しているのか。また，近年の高度な手技が要求される抗酸菌検査の精度管理がどの程度まで行われているのか。その現状を知ることは今後の抗酸菌検査の方向性を議論するうえできわめて重要である。これらの点について結核予防会結核研究所の御手洗聡先生から「わが国における抗酸菌検査の現状と精度保証」と題してご講演いただいた。阿部千代治先生から「今回の結核菌検査指針改訂のポイント」と題して抗酸菌検査の意義，安全管理，検査材料，検査の回数などの臨床現場での留意点や，CDC勧告に合致する迅速検査体制など，今回の改訂ポイントを総論的にご講演いただいた。検査感度の向上のために，本指針では検体の品質管理にも力点を置いた。結核の約8割を占める肺結核の喀痰検査の感度向上は治療・診断のみならず感染対策上も重要であり，そのためには検体検査として適切な喀痰を採取することが重要である。この点に関して臨床検査に従事し，現場の課題を熟知している京都大学医学部附属病院の樋口武史先生から「良質な検体とは―喀痰採取の方法等について―」と題して，採痰指導の有用性を中心にご講演をいただいた。抗酸菌検査の迅速性と感度の向上の観点から，培養は液体培養法が推奨され，同定は遺伝子検査法から，より迅速・簡便な免疫クロマトグラフィー法へと移行している。広島県環境保健協会の斎藤肇先生から「抗酸菌の培養・同定に関する最新情報」と題して，培養と菌種同定に関する最新の話題をご提供いただいた。核酸増幅法に代表される遺伝子検査法は今や結核診療において不可欠であり，培養感度に匹敵する新しい遺伝子検査法も登場している。東北大学病院の長沢光章先生から「遺伝子検査の現状と将来展望」と題して，最新の

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遺伝子検査法とその留意点についてご講演いただいた。今回の迅速検査体制構築の要である薬剤感受性検査法について，本指針では迅速性に優れる液体培地法を推奨した。順天堂大学医学部附属練馬病院の小栗豊子先生から「薬剤感受性成績を30日以内に報告するためには」と題して，液体培地を用いた迅速薬剤感受性検査の有用性とその課題についてご講演いただいた。結核菌検査は早期診断・早期治療や薬剤感受性検査結果に基づいた適正医療の普及などの診療上だけでなく，院内感染対策や結核対策のツールとしても必須であり，その精度向上は重要である。そして，現状の入院期間短縮への方向性を鑑みると，わが国においてもCDC勧告に沿った迅速検査体制の構築は急務であり，今回の「結核菌検査指針2007」は時宜を得た改訂といえる。結核菌検査の精度管理の向上と迅速性に向けての本シンポジウムでの議論はわが国の結核医療のさらなる前進に寄与するものと期待する。

1. わが国における抗酸菌検査の現状と精度保証

結核予防会結核研究所抗酸菌レファレンスセンター細菌検査科　御手洗　聡

結核を含む抗酸菌感染症の診断において，細菌学的検査が重要であることはよく認識されているが，その精度に関する臨床的関心は決して高いとは言えない。臨床的な関心の低さと実践上の困難性から，抗酸菌検査の精度保証活動は，内部精度管理を含めてあまり実施されていないのが現状と思われる。今回，結核菌検査指針が改訂されるにあたって，あらたに「精度保証」の項が設けられており，システムとしての精度保証が発展することが望まれる。ここでは，まず日本における抗酸菌検査と精度保証の現状について検討した。

わが国における抗酸菌検査は，そのほとんどが旧国立療養所等の院内検査室および衛生検査所（検査センター）で実施されているものと思われた。そこで，結核病床を有している病院および微生物検査実施を届け出ている検査センターを対象とし，抗酸菌塗抹，培養，同定，感受性検査，核酸増幅法にかかる一連の抗酸菌検査実施状況や精度保証活動内容を把握するためのアンケート調査を実施した。具体的には，病院および検査センター用に2種類の調査票を準備し，各設問に対して選択式の回答を得た。また，その際調査の信頼性のため，調査結果については一切匿名化し，施設名は公表しないことを文書にて明示した。2003年時点で結核病床を有している病院390施設および微生物検査実施を届け出ている検査センター397施設を対象として調査依頼を行い，それにより，最終的に291（74.6%）の病院検査室と288（72.5%）の検査センターから回答を得た。平均は73.6%（579/787）であった。

結果として，病院検査室の99.7%（290/291）が抗酸菌検査を実施していたが，塗抹から培養・同定・薬剤感受性検査および核酸増幅法をすべて自施設で実施していたのは27.1%（79/291）であった。塗抹・培養は80%以上の施設が実施していたが，同定や薬剤感受性検査を実施していたのは半数以下であった。検査センターについては288施設から回答を得たが，そのうち150施設（52.1%）では微生物検査を行っているものの，抗酸菌検査は実施されていなかった。他の138の検査センターでは1施設を除いて塗抹検査を実施しており，培養検査も87.0%（120/138）で実施されていた。しかしながら，薬剤感受性検査は65施設（47.1%）のみで実施されており，核酸増幅検査については47施設（34.1%）でのみ実施されていた（Table 1）。

病院検査室と検査センターの両者を合計すると，2002年の全国の検査実施数は，塗抹検査が143万件，培養が151万件，菌種同定が9.1万件，薬剤感受性検査が7.4万件，核酸増幅法が約60万件であった。アンケート回収率から，これが日本全国のおよそ4分の3を反映していると仮定すると，塗抹・培養検査は年間約200万件実施されていると考えられた。

Table 1 Type of laboratory and examinations performed (multiple answer)

Examination	Number of laboratory (%)	
	Hospital (n=243)	Commercial (n=138)
Smear microscopy	243 (100)	137 (99.3)
Culture	211 (86.8)	120 (87.0)
Species identification	122 (50.2)	77 (55.8)
Drug susceptibility testing	134 (55.1)	65 (47.1)
Nucleic acid amplification	96 (39.5)	47 (34.1)

続いて各検査法について検討した。塗抹検査については，迅速性のために直接法を実施している施設は病院と検査センターでそれぞれ70.8% (172/243) と81.0% (111/137) であった。一方，集菌法実施がそれぞれ40.3% (98/243) と29.9% (41/137) あり，両方を実施している施設もそれぞれ11.1%と10.9%であった。定期的な染色液のチェックは病院では33.0%，検査センターでは69.3%で行われていたが，病院では72.8% (177/243) で検査時染色コントロールを実施していないと回答した。塗抹検査の結果は，病院では97.5% (234/240)，検査センターでは80.3% (110/137) で24時間以内に報告されていた。

培養検査については，回答 (複数回答) を得た病院検査室206施設と検査センター118施設の全施設で固型培地を利用しており，病院では43.7% (90/206)，検査センターでも28.0% (33/118) で液体培地も使用されていた。塗抹陽性における培養陽性率が90%以下の施設が病院で49.7% (103/207)，検査センターで33.0% (38/115) あり，雑菌混入率が適正な値といわれている2～5%となっていたのは病院検査室で43.1% (90/209)，検査センターで39.0% (46/118) であった。培養陽性の結果報告までの期間が平均2週間以内であったのは，病院で15.3% (32/209)，検査センターで4.2% (5/118) であった。病院の96.6% (199/206) および検査センターの72.4% (76/105) で培養コントロールが実施されていなかった。

抗酸菌菌種同定検査については遺伝子ベースでの同定が推奨されているが，病院検査室の15.6% (19/122) と検査センターの75.8% (50/66) でいまだにナイアシンテストが実施されていた。これは臨床からの要求によるものと考えられた。

薬剤感受性検査時に標準株をコントロールとして同時に試験していた施設は病院で31.8% (41/129)，検査センターで43.6% (17/39) であった。感受性検査結果の報告は米国CDCの勧奨では1カ月以内となっているが，これを満足したのは，病院では17.3% (23/133)，検査センターでは18.8% (9/48) であった。

核酸増幅法についてみると，この当時多くの施設はアンプリコアを利用しており，陽性・陰性の反応コントロールを毎回実施しているのは病院で82.3% (79/96)，検査センターで96.2% (25/26) であった。

外部精度評価の実施についてTable 2に示した。病院検査室の60.0% (145/243)，検査センターの46.7% (64/137) が外部精度評価の実施なしと回答したが，塗抹検査では検査センターの40.9% (56/137) が，核酸増幅法では病院の34.6% (84/243) と検査センターの23.4% (32/137) が参加経験ありと回答した。核酸増幅法で外部精度評価の実施が比較的多かったのは，精度評価を行う研究会の存在と，検体の準備が比較的容易であることによるものと思われた。

精度保証の一環として検査室環境について質問した結果，細菌検査室を分離・独立させている施設は病院で92.6% (225/243)，検査センターで100% (138/138) であった。安全キャビネットの使用率は病院で80.2% (194/242)，検査センターで79.3% (107/135) であったが，薬剤感受性検査を実施する際の安全キャビネット使用はそれぞれ93.3% (125/134) と90.6% (58/64) であった。しかしながら，検査技師の健康診断を実施していないと回答した施設が病院で13.2% (31/235)，検査センターでも9.0% (12/133) あった。抗酸菌検査に関するトレーニングについても，定期的に参加しているのは病院では0% (0/226)，検査センターでも3.2% (4/124) であった。

2003年時点での抗酸菌検査実施状況について調査したところ，集菌法の実施が増加しており，液体培地での培養実施も進んでいた。この傾向は現在も続いていると考えられる。抗酸菌検査精度保証の実態は，内部精度管理・外部精度評価ともに不十分なものであり，特に塗抹，培養，薬剤感受性検査における精度管理用検査コントロールの実施は低率であった。外部精度評価は核酸増幅法では比較的高率に実施されていたが，他の検査については低率であった。これはパネルテスト等に関する具体的な方法が確立されていないことや，実施に必要な経費等の問題によるものと考えられた。また，検体の質の保証，検査コントロールの重要性の認識，トレーニング (初期あるいはリフレッシュ) に対する意欲なども不十分かと思われた。

Table 2 Implementation of external quality assessment (multiple answers)

Examination	Number of laboratory (%)	
	Hospital (n=243)	Commercial (n=137)
Smear microscopy	18 (7.4)	56 (40.9)
Culture	6 (2.5)	18 (13.1)
Species identification	9 (3.7)	14 (10.2)
Drug susceptibility testing	10 (4.1)	24 (17.5)
Nucleic acid amplification	84 (34.6)	32 (23.4)
No external assessment	145 (60.0)	64 (46.7)

精度保証の基本は，再検査やパネルテストによる現状の把握を実施し，その結果に基づいて改善活動を行い，再度テストを行って改善を確認し，これを一つのサイクルとして繰り返すことにある。抗酸菌検査は現在も広範に実施されており，感染症法の改正にも鑑みて，安全な検査環境と系統的な精度保証システムが必要と考えられた。

2. 今回の結核菌検査指針改訂のポイント

日本ベクトン・ディッキンソン株式会社　阿部千代治

1. 安全管理

結核菌検査にはエアロゾル発生頻度の高い操作が多いこともあり，濃厚な検査材料を毎日取り扱う検査技師の結核発病率は一般の人より数倍高い。結核菌検査に関するバイオセーフティマニュアルにみるように，結核菌群に属する菌はバイオセーフティレベル3，ほとんどの非結核性抗酸菌はレベル2に分類されている。また公布された感染症法では結核菌は4種病原体，多剤耐性結核菌は3種病原体に分類されており，結核菌検査はクラスⅡの安全キャビネットを備えたレベル2以上の設備のもとで行わなければならない。検査室内では専用のガウン，N95マスク，ディスポーザブル手袋を着用する。作業に当たってはエアロゾル発生の最も少ない方法を選択する。作業終了後に安全キャビネットや実験台を噴霧消毒し，その後殺菌灯を点灯する。使用後の材料や器具は高圧蒸気滅菌処理をする。またバイオテロを防ぐために分離された結核菌は鍵のかかる部屋または保管庫で保存しなければならない。検査に従事する検査技師は採用時または細菌検査室への配置前にQuantiFERON-TB検査を受ける。ハイリスクグループでは年2回の定期健診を受ける。

2. 検査の方法

（1）検査材料および検査回数

提出された喀痰が適切なものかどうかは検査の精度を保持するうえで重要である。第2章「検査材料」の項に「採痰指導」が加えられた。8～24時間間隔で3回喀痰を採取，少なくとも1回は早朝痰を採取する。但し初回の検査で2＋以上であれば3回の検査は必要ない。幼児や高齢者などで喀痰の排出が困難な場合に誘導喀痰や胃液の検査をすることは有効である。3回の塗抹検査が陰性の場合に塗抹および培養検査を行った日とは別の日に気管支鏡を用いた検査を保険診療で行うことができる。

（2）塗抹検査

均等化・遠心集菌材料の塗抹を勧める。本指針では剝離対策のために剝離防止処理したスライドの使用を勧めている。「新結核菌検査指針2000」で推奨したことで塗抹検査に蛍光法を取り入れた施設が増加した。蛍光染色として，オーラミンO染色に，新たにアクリジンオレンジ染色法が取り上げられた。オーラミンは菌体周囲の脂質を染色するのに対し，アクリジンオレンジは核酸を染色する違いがある。

（3）検体の前処理

前処理の目的は検査材料を消化・均等化し，含まれる雑菌を殺し抗酸菌のみを選択的に培養することにある。NALC-NaOH処理のみでは汚染が除去できない検体があること，1つの検体を一般細菌の培養にも使用するケースがあることなどから，セミアルカリプロテアーゼによる消化・均等化が推奨されている。また遠心補助剤を使用した前処理法など新たに開発された前処理法の有効性が証明されており，この改訂書に加えられた。

（4）培養検査

迅速な検査結果の報告が求められている。発育インジケーター付液体培地として，KRD培地が加えられた。抗酸菌自動培養システムとして，BACTEC MGIT 960，バクテアラート3D，BACTEC 9000自動血液培養システムが取り上げられている。本検査指針では初回分離に液体培地と卵培地（固形培地）を1本ずつ用いることを勧めている。しかし，検査室の受容力や液体培地の価格の問題があることから施設内で検討する。3回の培養に液体培地のみを用いるのでなく，1回は固形培地を用いる。

（5）分離抗酸菌の鑑別・同定

結核菌と非結核性抗酸菌の早期の鑑別は適切な患者管理と治療のうえで重要である。結核菌群特異抗原をイムノクロマトグラフィーにより検出するキャピリアTBは簡便であり，液体培地と併用することにより迅速かつ簡便に結核菌と非結核性抗酸菌を鑑別でき有用である。液体培地の導入により非結核性抗酸菌の分離頻度が高まった。現在100種以上の抗酸菌種が報告されており，従来からの培養・生化学的性状や市販の遺伝子診断キットでそれらを同定することは不可能である。複数の遺伝子の塩基配列を決定し同定する方法が報告されている。検査

可能な施設に同定を依頼する必要がある（第5章「抗酸菌の同定」を参照）。

（6）遺伝子検査

初回診断時の3日間の塗抹および培養検査に加え，核酸増幅法による検査を1回保険診療で行うことができる。喀痰塗抹陽性例では患者管理のうえで結核か非結核性抗酸菌かを早急に鑑別する必要があり，検体入手後1～2日で結果が得られる核酸増幅法による検査は有効である。「新結核菌検査指針2000」の出版後に体外診断薬として承認，保険収載されたコバスTaqMan MTB，TRCRapid M.TB，結核菌群リファンピシン耐性遺伝子同定検査が改訂書に加えられた。"非結核性抗酸菌症の診断において核酸増幅法はあくまで補助的診断であり診断基準に含めない"とする非結核性抗酸菌症対策委員会の見解から，塗抹陰性の場合に*M. avium*または*M. intracellulare*の検査は行わない。

（7）薬剤感受性検査

日本結核病学会が小川培地を用いる比率法を承認し，標準法として使っている。小川培地を用いる比率法は結核菌ならびに*M. kansasii*の抗結核薬に対する感受性検査法である。*M. kansasii*以外の非結核性抗酸菌について，小川培地を用いる薬剤感受性検査は行わない。これは感受性検査結果と臨床応答との間に関連はみられないからである。

薬剤感受性検査は最も精度管理の難しい検査である。米国のCLSIは精度を保持するために寒天培地または液体培地の使用を勧めている。わが国でも検査精度に加え迅速性が重視され，液体培地が取り入れられつつある。この改訂書でもBACTEC MGIT 960 AST，マイクロプレートを用いるMIC測定法などが取り上げられた。

（8）精度保証

検査精度をチェックするために精度管理テストは必須である。日常行う内部精度管理に加え，定期的に外部施設による精度管理も取り入れる必要がある。実施している施設と連絡先が記述された。

3. 今求められている検査

1980年代の中期から1990年代の初期にかけて米国の結核罹患率が急増した。特にHIV感染に伴う結核は診断が難しいうえに病気の進行が速いこともあり，米国のCDCは検査室に迅速な結果報告を求めた。その目標は，塗抹の結果を24時間以内・培養の結果を21日以内・感受性結果を30日以内に担当医に報告するというものである。現在入院期間の短縮が求められている。そのためには感受性結果をできるだけ早く報告する必要がある。

検査結果は患者管理や治療に結びつくことから迅速性に加え高い精度が要求される。精度の善し悪しは新たに耐性菌を作ることにもつながる。定期的な内部精度管理が求められる。

3. 良質な検体とは—喀痰採取の方法等について—

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はじめに

良質な喀痰の採取方法として採痰指導を取り上げ，その有用性について解説する。また「結核菌検査指針2007」の第2章「検査材料」，および第3章「塗抹検査」の変更点についても併せて概説する。

採痰の原理

細気管支などの末梢付近にある喀痰を体外に排出するには，その気管支の奥（肺胞側）に空気を送り込み，腹筋などを利用することにより空気を圧縮して喀痰を気管支のほうへ押し上げて排出する方法と，気管支を拡張させて肺胞まで空気を十分に送り込み，気管支内壁に付着した喀痰を剝ぎ取るようにして排出する方法がある。いずれの方法も，深呼吸によって肺全体の容積を拡張させ十分に空気を取り入れることがポイントである。肺のすみずみまで十分に空気が取り込まれた状態から腹筋を一気に収縮させ咳嗽させる。このとき，約200 cmH_2Oに高められた気道内圧で気管に生じる気流速度は，約1,000 km/hに達することが知られており，これはジェット気流に匹敵する速度である。この気流を効率よく利用することで，喀痰は気道へと押し上げられ体外に排出される(Fig.)。この原理に基づいた呼吸運動を行わなければ容易に痰を排出することはできない。具体的には痰を排出させる手段としてスクイージングなどの理学療法を習得することがポイントになる。

採痰指導の有用性

2000年9月1日から2001年8月31日の1年間に呼吸器内科外来を受診した患者163人（指導無群）と2001年9月1日から2002年8月31日の1年間に同外来を受診した患者161人（指導有群）から採取した喀痰について

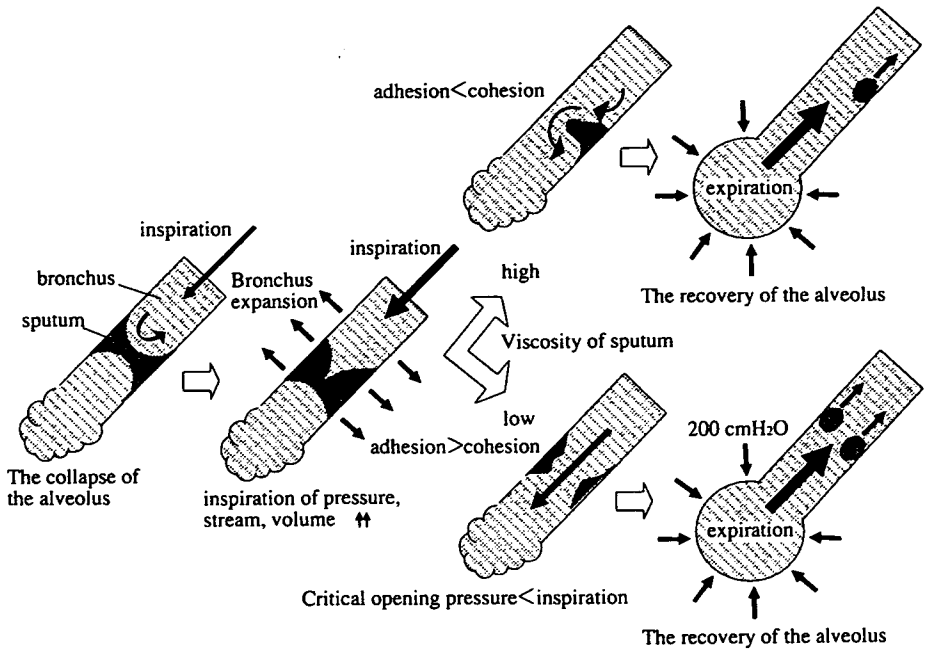

Fig. The mechanism of expectoration

採痰指導の有用性を評価した。その結果，M1およびP1喀痰は，指導無群では21.5%，21.5%，指導有群では8.1%と36.6%であった。M2，P2，P3喀痰は両群で大きな差を認めなかった。塗抹陽性率は，指導無群10.4%，指導有群21.1%であった。M2，P1，P2の肉眼的性状別での陽性率は，指導無群で11.1%，11.4%，30.8%で，指導有群では17.6%，28.8%，26.3%であった。これらの塗抹陽性患者（指導無群17名，指導有群34名）での空洞の有無，病巣の拡がりから判定した胸部X線所見で，指導無群は指導有群に比べて空洞型で中～高範囲の病巣を有する重症型を多く含んでいた。この結果から採痰指導有群で見られた喀痰の肉眼的性状や塗抹陽性率の向上は，患者重症度の差に由来する可能性は否定された。採痰指導は，塗抹陽性率を向上させるのに有用であることが証明された。

「結核菌検査指針2007」の変更点

第2章「検査材料」の変更点

検体の品質管理が重要であることを強調し，特に喀痰採取の方法として採痰指導の項目を付記した。検体および菌株の輸送に関する項目を第8章「精度保証」へ移動した。

第3章「塗抹検査」の変更点

塗抹標本の剝離対策として推奨していたタンパク液に替え，MASコートやAPS（シラン）コートなどの剝離防止処理したスライドガラスの使用を推奨した。染色はオーラミンO蛍光染色法を推奨した。ただし，オーラミンOは発癌性物質であるため，試薬の安全性，安定性が優れているアクリジンオレンジ染色法を新たに追加した。塗抹結果の記載法の一部を訂正した。

ま と め

結核の確定診断には，感染病巣から採取された検体を用いて細菌学的に結核菌を証明することが重要である。喀痰は品質管理上最も重要かつ難しい検体であり，医療従事者は患者の病態を反映した検体が採取できるように最大限努力する必要がある。したがって，採痰指導などのいろいろなアプローチを行い，積極的に喀痰採取に関与することが抗酸菌検査の精度向上に大きく関与する。

4. 抗酸菌の培養・同定に関する最新情報

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はじめに

抗酸菌の分離培養は，塗抹染色よりも感度が高く菌を検出でき，また分離菌の鑑別・同定や薬剤感受性試験などを行うことができる。以下にこれらのうち，分離培養法ならびに従来法による分離菌の鑑別・同定法について，最新情報をまじえながら述べる。

Ⅰ．分離培養法

〔A. 前処理法〕

喀痰などの汚染検体を消化・均質化し，混在する抗酸菌以外の細菌を殺して（decontamination），抗酸菌のみを選択的に培養する方法で，NALC-NaOH法が広く用いられてきた。最近では，検体をあらかじめセミアルカリプロテアーゼ（商品名：スプタザイム，プレソルブ）で溶解・均質化し，NALC-NaOH溶液が効率よく作用し，かつ，分離培地の雑菌汚染の低減を図ろうとする方法が推奨されている。分離培養を行うにあたっては，所定濃度の水酸化ナトリウム溶液を用い，かつ検体の処理時間に留意すべきである。

（1）N-アセチル-L-システイン・水酸化ナトリウム（NALC-NaOH）法

NALC-NaOH（終末1～2%）溶液で喀痰を消化・汚染除去し，リン酸緩衝液で希釈後，3,000×g，20分遠心し，沈渣を少量の同種緩衝液に再浮遊させて，培地へ接種する方法である。NALC-NaOH液に加えてあるクエン酸ナトリウムは喀痰中に存在するであろうNALCの不活化重金属イオンをキレートし，NALCに安定性を与えるものである。本法は米国CDCで開発され，推奨される方法である。

（2）抗酸菌検出用キット“ニチビー”（ニチビー法）

CC-Eキット（前処理液）と遠心集菌剤（K-8）よりなる。CC-EキットはCC-E液（喀痰膨潤剤加2%NaOH）とCC-E助剤（長期安定型NALC溶液）からなり，喀痰処理時間の短縮と処理能の増強とを図ったものである。処理喀痰をリン酸緩衝液で希釈し，K-8（塩化ポリアルミニウム）を加えると，陰性荷電の菌体と陽性荷電のK-8とが凝集物を作るため，菌を低速（1,600×g），短時間（5分）で濃縮でき，沈渣をリン酸緩衝液に再浮遊後，培地へ接種する。

われわれが本法およびNALC-NaOH法で処理した喀痰380検体をMGIT 960システムおよび2%小川培地で培養した際の抗酸菌検出成績は，両検体処理法間に有意差を認めなかった。

（3）抗酸菌検査用喀痰前処理キット セントラップMB「ニッスイ」

喀痰をA試薬（NALC-NaOH溶液）で処理後，B試薬（吸着担体生成試薬）を加え，生じた白濁不溶性沈殿物を2,000×g，10秒間遠心し，沈渣をC試薬（懸濁試薬）へ再浮遊し，培地へ接種する。

〔B. 培地〕

わが国で用いられている抗酸菌の分離培地は，固型培地としては小川培地，液体培地としてはMGITが広く用いられているが，マイクロコロニーの早期検出と形態観察，集落形成単位（CFU）の測定には寒天をベースとした平板培地が有用である。この場合，CO_2ふらん器内での培養が必要である。

われわれは，NALC-NaOH処理喀痰431例についてのKRD培地“ニチビー”（液体培地）による抗酸菌分離培養成績がBACTEC 960 MGITシステムと遜色なく，小川培地よりも有意にすぐれていることを報告している。

初診時における3回連続検痰に用いる培地の組合せは検査施設の事情により異なるが，発育支持能と迅速性にすぐれた液体培地と固型培地（2%小川培地）との併用が望ましく，以下のような組合せが考えられよう。

①3回とも液体培地と小川培地の併用，②2回は液体培地と小川培地の併用・1回は小川培地のみ，③1回は液体培地と小川培地の併用・2回は小川培地のみ。他方，液体培地使用困難な施設では小川培地を主軸とし，塗抹陰性であっても結核の疑いが強い場合，あるいは塗抹陽性であっても治療上あるいは疫学上，結核菌を早期に検出・同定することが強く望まれる場合には液体培地を併用することが望ましい。

Ⅱ．同 定

抗酸菌を迅速に同定することは，患者の治療方針の決定のためのみならず，疫学面からもきわめて重要である。

〔A. 抗酸菌種〕

承認・提案されている抗酸菌種をヒトに対する起病性別にみるとTable 1に示すようである。

1. *Mycobacterium tuberculosis* complex（結核菌群）

M. tuberculosis, *M. bovis*, *M. africanum*, *M. microti*に加え，近年*M. canettii*（van Soolingen D, et al. 1997），*M. pinnipedii*

Table 1 Description of mycobacterial species

Category group			Involvement in human disease					
			Common	Rare		None		
Slowly growing mycobacteria	TB complex		***M. tuberculosis***	*M. microti*	*M. pinnipedii*			
			M. bovis*	*M. caprae*				
			M. africanum*	*M. canettii*				
	Nontuberculous mycobacteria	I*	***M. kansasii***	*M. simiae*				
			M. marinum	*M. asiaticum*				
		II	***M. scrofulaceum***	***M. gordonae***	*M. interjectum*	*M. botniense*		
			M. xenopi*	***M. heckeshornense***	*M. nebraskense*	*M. cookii*		
			M. ulcerans*	***M. intermedium***	*M. palustre*	*M. doricum*		
				M. lentiflavum	*M. parascrofulaceum*	*M. farcinogenes*		
				M. shinshuense	*M. parmense*	*M. hiberniae*		
				M. szulgai	*M. saskatchewanense*	*M. kubicae*		
				M. bohemicum		*M. tusciae*		
		III	***M. avium* subsp. *avium***	***M. branderi***	***M. triplex***	*M. avium* subsp. *silvaticum*		
			M. intracellulare	***M. celatum***	*M. avium* subsp. *paratuberculosis*	*M. avium* subsp. *hominissuis*		
			M. malmoense*	***M. genavense***		*M. gastri*		
				M. haemophilum	*M. conspicuum*	*M. lepraemurium*		
				M. nonchromogenicum	*M. heidelbergense*	*M. montefiorense*		
				M. shimoidei	*M. lacus*	*M. shottsii*		
				M. terrae	*M. sherrisii*	*M. triviale*		
Rapidly growing mycobacteria		IV	***M. abscessus***	***M. fortuitum* subsp. *acetamidolyticum***	*M. peregrinum*	*M. agri*	*M. hassiacum*	
			M. chelonae		*M. porcinum*	*M. aichiense*	*M. hodleri*	
			M. fortuitum	***M. goodii***	*M. senegalense*	*M. album*	*M. holsaticum*	
				M. mageritense	*M. septicum*	*M. alvei*	*M. komossense*	
				M. thermoresistibile	*M. smegmatis*	*M. aurum*	*M. madagascariense*	
				M. boenickei	*M. wolinskyi*	*M. austroafricanum*	*M. moriokaense*	
				M. brisbanense		*M. brumae*	*M. murale*	
				M. canariasense		*M. chitae*	*M. obuense*	
				M. conceptionense		*M. chlorophenolicum*	*M. parafortuitum*	
				M. elephantis		*M. confluentis*	*M. phlei*	
				M. houstonense		*M. chubuense*	*M. poriferae*	
				M. immunogenum		*M. diernhoferi*	*M. pulveris*	
				M. manitobense		*M. duvalii*	*M. rhodesiae*	
				M. massiliense		*M. fallax*	*M. sphagni*	
				M. mucogenicum		*M. flavescens*	*M. tokaiense*	
				M. neoaurum		*M. frederiksbergense*	*M. vaccae*	
				M. neworleansense		*M. gadium*	*M. vanbaalenii*	
				M. novocastrense		*M. gilvum*		
				M. parmense		*M. hackensachense*		

(Boldface) Previously reported mycobacteria associated with human diseases in Japan.
*Mycobacteria frequently involved in human disease in some particular counties or areas. *M. leprae* can not be cultured *in vitro*.
"*M. visibilis*" is usually difficult in cultivation.
*Runyon's classification.

(Hajime Saito, 2007).

(Cousins DV, et al. 2003), *M. caprae* (Prodinger WM, et al. 2005) が報告されている。いずれもヒトに対して病原性を有する。

2. Nontuberculous mycobacteria (非結核性抗酸菌)

主として，肺結核類似症の原因菌として，稀に分離される抗酸菌について，自験例を中心に簡単に述べる。

(1) I群菌

新種として *M. intermedium* (Meier A, et al. 1993) が報告されているが，集落は光発色性ではなく，暗発色性で，II群菌である。著者らもその1例を報告している。

(2) II群菌

① *M. lentiflavum* (Springer B, et al. 1996)：脊椎椎間板炎病巣から分離・命名された。岩本・著者らは肺疾患患者の喀痰・気管支洗浄液から分離された8株を本菌と同定し，これらを3遺伝子型に分類した。

② *M. heckeshornense* (Roth A, et al. 2000)：*M. xenopi* にきわめて近似した表現型性状を有し，またDDHテストでは *M. xenopi* と同定される。著者らは，わが国で分離・保存されている *M. xenopi* 12株中6株を *M. heckeshornense* と同定した。両菌種はアリルスルファターゼテスト (3日法)，16S rDNA配列決定により鑑別可能である。

③ *M. ulcerans* (MacCallum P, et al. 1948) と *M. shinshuense* (Tsukamura M, Mikoshiba H, 1982)：*M. shinshuense* と *M. ulcerans* の異同性については未だ明らかでない。共に毒